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Mouse psychosocial stress reduces motivation and cognitive function in operant reward tests: a model for reward pathology with effects of agomelatine

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Short title: Stress-induced decreased motivation in mice

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Abstract

A major domain of depression is decreased motivation for reward. Translational automated tests can be applied in humans and animals to study operant reward behaviour, aetio-pathophysiology underlying deficits therein, and effects of antidepressant treatment. Three inter-related experiments were conducted to investigate depression-relevant effects of chronic psychosocial stress on operant behaviour in mice. (A) Non-manipulated mice were trained on a complex reversal learning (CRL) test with sucrose reinforcement; relative to vehicle (VEH), acute antidepressant agomelatine (AGO, 25 mg/kg *p.o.*) increased reversals. (B) Mice underwent chronic social defeat (CSD) or control handling (CON) on days 1-15, and were administered AGO or VEH on days 10-22. In a progressive ratio schedule motivation test for sucrose on day 15, CSD mice made fewer responses; AGO tended to reverse this effect. In a CRL test on day 22, CSD mice completed fewer reversals; AGO tended to increase reversals in CSD mice associated with an adaptive increase in perseveration. (C) Mice with continuous operant access to water and saccharin solution in the home cage were exposed to CSD or CON; CSD mice made fewer responses for saccharin and water and drank less saccharin in the active period, and drank more water in the inactive period. In a separate CSD cohort, repeated AGO was without effect on these home cage operant and consummatory changes. Overall, this study demonstrates that psychosocial stress in mice leads to depression-relevant decreases in motivation and cognition in operant reward tests; partial reversal of these deficits by AGO provides evidence for predictive validity.

Keywords: depression; decreased motivation; animal model; operant; circadian rhythm; agomelatine

1. Introduction

Depression is a major and heterogeneous neuropsychiatric disorder. Core psychopathological symptoms are depressed mood, diminished interest in activities, and increased fatigue, together with a number of common symptoms including weight loss or gain, psychomotor agitation or retardation, insomnia or hypersomnia, and reduced concentration and attention (DSM-5, 2013; ICD-10, 1994). The recent Research Domain Criteria (RDoC) project (Cuthbert, 2015; Cuthbert and Insel, 2013) proposes a classification system that takes diagnostic systems into account but also identifies the importance of understanding individual psychopathologies, which have been organised into domains: for example, the Positive valence systems domain, which includes approach motivation, responsiveness to reward and reward learning; and the Arousal/modulatory systems domain which includes arousal, biological rhythms and sleep-wake (Cuthbert and Insel, 2013). Integrating RDoC with depression symptomatology, the Positive valence systems domain is clearly relevant to diminished interest and the Arousal/modulatory systems domain is clearly relevant to insomnia or hypersomnia. Depression is not well-understood in terms of aetio-pathophysiology, and patients with different symptom combinations are also likely to differ at the pathophysiological level. The RDoC approach is conducive with translational research aimed at increasing understanding of disorder pathophysiology. This includes evidence obtained from animal models that combine manipulations with aetiological validity with behavioural readout tests with face validity for specific psychopathologies (Pryce and Seifritz, 2011).

Chronic psychosocial stressors are major aetiological risk factors for depression (Kendler *et al.*, 2003; Kessler, 1997). In mice, one form of environmental manipulation proposed to model aspects of human psychosocial stress is chronic social defeat (CSD). It comprises 10-15 days of continuous intruder status in the home cages of different dominant mice but protected by a divider, with brief daily experience of actual physical attack and defeat (Golden *et al.*, 2011; Kudryavtseva *et al.*, 1991). We and others could demonstrate that CSD leads to increased sensitivity to and impaired coping with aversive stimuli (e.g. (Azzinnari *et al.*, 2014). Furthermore, CSD has been reported to result in decreased preference for gustatory reward, namely sweet-tasting sucrose solution, over water in the two-bottle test of consummatory behaviour (Krishnan *et al.*, 2007) . Somewhat in contrast, in human depression, patients do not exhibit reduced subjective pleasure when given a rewarding stimulus (Dichter *et al.*, 2010). However, when required to exhibit high effort to obtain reward, depressed patients are less motivated to do so (Sherdell *et al.*, 2012). This evidence that, in depression, positive-valence processing is reduced at the motivational level rather than the consummatory level, highlights the need for animal models of stress-induced impairment of reward motivation and anticipation. These processes can be best-studied using behaviour-outcome

(operant) tests. ~~To our knowledge there is currently no published report on the effects of CSD on operant reward-directed behaviour in mice.~~

In order to investigate reward motivation *per se*, a single operant stimulus can be used with reinforcement earned according to a specific schedule. In animal studies, the reinforcer typically takes the form of palatable food. The progressive ratio schedule (PRS) test requires the subject to make an increasing number of responses to obtain successive rewards and is therefore sensitive to assessing the motivation for effortful responding for reward. By conducting the PRS test with the animal subject close to hunger satiety, the anticipated palatability of the sucrose-pellet reward becomes more important relative to its calorific value, thereby increasing the test's sensitivity for assessing reward motivation. For example, in adult rats it has been demonstrated that early life stress leads to decreased responding for reward in a PRS test (Leventopoulos *et al.*, 2009). Using tests comprising two operant stimuli that need to be discriminated between in order to obtain reinforcement, then a number of further depression-relevant reward processes can be investigated. When depressed patients are assessed in discrimination tests that require low effort to obtain reward, typically either symbolic (emoticon e.g. smiling face) or monetary, they do not differ from healthy subjects in terms of accuracy of responding (Taylor Tavares *et al.*, 2008). However, their responding is characterised by high sensitivity to error feedback: when depressed patients make an error and therefore fail to receive reinforcement on a trial, they are more likely to make an incorrect decision on the subsequent trial (Elliott *et al.*, 1996; Elliott *et al.*, 1997). The probabilistic reversal learning (PRL) test assesses reward-directed decision making under conditions of accurate and misleading reinforcement/feedback (Chamberlain *et al.*, 2006; Cools *et al.*, 2002; Evers *et al.*, 2005; Jocham *et al.*, 2009). The test comprises reversal learning, which requires responding to regular shifts in the contingencies - reward, non-reward - between the two operant stimuli and reinforcement and, superimposed on this, at a certain probability correct responses are not rewarded whereas incorrect responses are rewarded. The proportion of non-rewarded correct responses on which the subject shifts (to the incorrect stimulus) on the next trial, gives a measure of negative feedback sensitivity (NFS). High NFS is indicative of under-estimation of reward probability and is increased in depressed patients (Taylor Tavares *et al.*, 2008). Recently, rodent automated operant PRL tests have been developed, firstly for rat (Bari *et al.*, 2010) and then in our laboratory for mouse (Ineichen *et al.*, 2012). In order to ensure that motivation for sucrose-pellet reinforcement is sufficient for rodents to engage in this demanding test, some food deprivation is essential. In the mouse PRL test, given that misleading feedback is restricted to non-rewarded correct responses (without any rewarding of incorrect responses), we refer to it here as a complex reversal learning (CRL) test. Non-manipulated mice exhibit: (1) reward-stay behaviour consistent with accurate monitoring of the average reward probability at each stimulus; (2) low perseveration and high NFS consistent with accurate monitoring

of expected and unexpected non-reward; (3) reduced reversals achieved relative to performance in a simple reversal learning test without any non-rewarded correct responses (Ineichen *et al.*, 2012).

Agomelatine (AGO; S 20098) is an agonist at melatonergic (MT_1/MT_2) receptors and an antagonist at serotoninergic 2C (5-HT_{2C}) receptors, and was recently approved in Europe for the treatment of major depression (de Bodinat *et al.*, 2010; Kennedy *et al.*, 2014). During its preclinical development, using repeated dosing, AGO was shown to be efficacious in several rat and mouse models relevant to depression domains (de Bodinat *et al.*, 2010). These include reversal of decreased saccharin consumption induced by chronic mild stress (Papp *et al.*, 2003), reversal of specific learned helplessness (Bertaina-Anglade *et al.*, 2006), antagonism of increased anxiety and decreased grooming induced by repeated corticosterone administration (Rainer *et al.*, 2012), and antagonism of increased anxiety, immobility in the forced swim test, and altered circadian rhythm of activity and sleep-wake pattern induced by prenatal restraint stress (Mairesse *et al.*, 2013; Marrocco *et al.*, 2014; Morley-Fletcher *et al.*, 2011). In clinical trials, therapeutic efficacy of AGO is increased relative to placebo in moderate to severe depression (Kennedy *et al.*, 2014). When the core symptom/domain of interest-pleasure was measured using a self-report questionnaire, in depressed patients efficacy of AGO on anhedonia was increased relative to venlafaxine (Di Giannantonio and Martinotti, 2012; Martinotti *et al.*, 2012). In terms of operant tests of reward processing, whilst there are no published studies with AGO to-date, a 5-HT_{2C} antagonist compound was demonstrated to improve reversal learning, in part by decreasing perseveration, in a rat model (Boulougouris *et al.*, 2008).

The overall aim of the present study was to establish a mouse model of psychosocial stress-induced impaired reward processing and to investigate its predictive validity with the antidepressant AGO. Three inter-related experiments were conducted: (1) Investigation of the effects of acute administration of AGO, melatonin and a 5-HT_{2C} antagonist on behaviour of control mice in the complex reversal learning test, to establish an effective AGO dose and its mechanism of action. (2) Investigation of the effects of 15-day CSD (Azzinnari *et al.*, 2014) on operant behaviour in a progressive ratio schedule test and in simple and complex reversal learning tests, and of the effects of repeated AGO administration on CSD-induced deficits. (3) Investigation of the effects of 15-day CSD on the amount and circadian distribution of operant responding for saccharin solution versus water in a home cage setting, and of the effects of repeated AGO administration on CSD-induced deficits.

2. Experimental procedures

2.1. Animals

Experiments were conducted with adult male C57BL/6J (BL/6J) mice bred in house, maintained in littermate pairs, and aged 10 weeks and weighing 26-28 g at study onset. Male CD-1 mice (Janvier, Le

Genest-Saint-Isle, France) used for social stress were aged 8 months, weighed 40-45 g, were ex-breeders, and caged singly. Mice were maintained on a reversed light-dark cycle (light off 07:00-19:00 h). Standard home cages were type 2L and maintained in an individually-ventilated caging system. One study was conducted using IntelliCages (see below). Both cage types contained sawdust, tissue bedding and a sleep igloo. Temperature was maintained at 22°C and humidity at 50-60%. The standard diet was Complete Pellet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water, both available continuously unless otherwise stated below. BL/6J mice were handled on 5 days at study onset. The studies were conducted under a permit (170/2012) for animal experimentation issued by the Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice studied and any unnecessary stress to those mice that were studied.

2.2. Experimental design

Three studies (A, B, C) were conducted, each with a different cohort of naive mice (Figure 1). The first stage of each study was operant training for reinforcement with sucrose-pellet (A, B) or saccharin-solution and water (C). In Study A (N=24), we investigated the effects of acute agomelatine (AGO), melatonin and S32006, a 5-HT_{2C} receptor antagonist, on behaviour in a complex reversal learning (CRL) test in otherwise non-manipulated mice. In Study B (N=44), we investigated the effects of chronic social defeat (CSD, days 1-15, versus control handling (CON)) and AGO (days 10-22, versus vehicle (VEH)) on behaviour in a progressive ratio schedule (PRS) test (day 8 and day 15), a simple reversal learning (SRL) test (day 19) and the CRL test (day 22). Study C comprised two experiments in IntelliCage: Firstly, we investigated the effects of CSD (days 1-15, versus CON) (N=20) on appetitive and consummatory behaviour towards water and saccharin solution during the dark- and light-phases of the circadian cycle. Second, in a new cohort (N=16) the effects of AGO (days 7-20, versus VEH) on these same measures were studied in CSD mice (days 1-15). With respect to the timeline of behavioural tests, in Study B, the two PRS tests allowed for assessment of development of CSD effects on reward motivation; this test is conducted in the absence of food deprivation and therefore no food deprivation was necessary during CSD (see below). The SRL and CRL tests allowed for assessment of post-CSD effects on these respective reversal learning challenges; food deprivation is required for these tests and this was an important factor in conducting these tests after completion of CSD. With respect to the timeline of agomelatine administration, in Studies B and C the aim was to initiate agomelatine administration at the mid-point of CSD (day 10 in Study B to allow for pre-drug testing of CSD effects in the progressive ratio schedule test on day 8, and day 7 in Study C) so that its effects on reversal - rather than prevention - of CSD effects could be investigated. The duration of agomelatine administration of 13-14 days was based on previous studies demonstrating reversal of

stress-induced behavioural effects after 2 weeks of agomelatine administration e.g. recovery of sucrose drinking in rats exposed to chronic mild stress (Papp *et al.*, 2003).

FIGURE 1 ABOUT HERE

2.3. Chronic social defeat

Our refined protocol for chronic social defeat is described in detail elsewhere (Azzinnari *et al.*, 2014). As in the majority of CSD protocols (see Golden et al., 2011; Azzinnari et al., 2014) the strain used as the stressor was CD-1. Ex-breeder males of this strain attack with a short latency and are generally larger than BL/6 males, thereby allowing for reliability in terms of the direction of the dominant-subordinate relationship. Also, CD-1 mice continue to attack when BL/6 mice display submissive behaviours, such that the latter experience lack of social control. In Study B, each BL/6J (CSD) mouse was placed singly in the 2L home cage of a CD-1 mouse, separated by a transparent, perforated divider. The CSD mouse was placed in the same compartment as the CD-1 mouse for either a cumulative total of 60-s physical attack (chase, wrestle, bite) or 10 min maximum. To prevent bite wounds, the lower incisors of CD-1 mice were trimmed every third day across CSD. The CSD mouse x CD-1 mouse pairings were rotated so that CSD mice were placed in the home cage of and confronted with a novel CD-1 mouse each day. This CSD procedure was conducted for 15 days, between 14:00-16:00 h. From day 16 until the end of operant testing, each CSD mouse remained in one cage with the same CD-1 mouse, without further attacks. The rationale for this was that following 15 proximate exposures to CD-1 mice, distal exposure to such a mouse should continue to represent a stressor until the end of the experiment. Control (CON) mice remained in littermate pairs, the standard condition in our laboratory, and were handled and weighed daily. For details of the CSD procedure in Study C, see sub-section IntelliCage appetitive and consummatory behaviour.

2.4. CSD effects on feeding homeostasis and appetite hormones

Given that the reversal-learning operant tests require food deprivation (Ineichen *et al.*, 2012), and that CSD is known to increase food intake and alter blood levels of appetite-regulating hormones in a pro-feeding direction (Kumar *et al.*, 2013; Patterson *et al.*, 2013), for Study B it was necessary to establish the effects of our CSD protocol on home-cage food intake and appetite hormone levels. In a separate experiment with naive mice, daily intake of food pellet was measured in 11 littermate pairs for 5 days prior to allocation to CSD (N=12) and CON (N=10) and measurement of daily intake of food pellet across the 15 days. For mice in pairs, pellet consumption per mouse was estimated by dividing the total amount of pellet eaten by two and adjusting for respective body weights. On day 16, mice were decapitated and trunk blood collected into 500 µl tubes coated with EDTA (Microvette, Sarstedt), and 4-2-aminoethyl-benzenesulfonyl fluoride (Sigma) was added at 1 mg/ml. The blood

was centrifuged at 3000 rpm for 15 min at 4°C, the plasma was removed to Eppendorf Protein LoBind tubes, and acidified with hydrochloric acid to a final concentration of 0.05 N. Plasma samples were stored at -20°C until determination of leptin and acylated ghrelin using ELISA kits according to the manufacturer's protocol (Mouse Leptin EZML-82K, Rat/Mouse Ghrelin (active) EZRGRA-90K, Merck Millipore).

2.5. Operant training and testing

Controlled feeding and body weight. In Studies A and B, body weight (BW) and food intake of BL/6J mice were measured every day for one week and the mean values were calculated and taken as baseline for each mouse. To ensure that mice were motivated for operant training, BW was reduced to 90-95% baseline by controlling daily pellet allowance. In Study B, at 5 days prior to and throughout CSD, mice were given sufficient daily pellet to return to and maintain 100% baseline BW. They were then maintained at baseline BW for progressive ratio schedule testing and reduced to 95% baseline BW for reversal learning tests.

Operant chambers. Modular operant chambers (TSE Systems, Bad Homburg, Germany, (Ineichen *et al.*, 2012)) were used for Studies A and B. Briefly, each chamber contained one (progressive ratio schedule test) or two (reversal learning tests) operant nose-poke apertures and one feeder that delivered one sucrose pellet per reinforcement (14 mg Dustless Precision Pellets, TSE Systems). Correct and incorrect nose-pokes and pellet retrieval were detected via infrared beam breaks in the respective apertures. A loudspeaker above the feeder emitted a tone to signal reward delivery. After each session the chambers were cleaned with 70% ethanol.

Training on fixed-ratio 1 (FR1) operant reinforcement. In Studies A and B, mice were conditioned to nose poke on a fixed-ratio 1 (FR1) schedule for sucrose pellet reinforcement during five sessions per week for 1-2 weeks, using the training stages detailed in (Ineichen *et al.*, 2012). The basic settings were: trial onset was indicated by illumination of the nose-poke aperture(s); one response (FR1) initiated tone and pellet delivery, and following pellet retrieval a 2.5 s inter-trial interval (ITI) was initiated; the session terminated after 40 reinforcements or 30 min.

Progressive ratio schedule (PRS) test. The PRS test was used in Study B, and conducted as described elsewhere (Ineichen *et al.*, 2012; Bergamini *et al.*, in press). Briefly, only one nose-poke aperture was used. The maximum session duration was 40 min. Session parameters were: required number of responses on first trial = 1, number of consecutive trials for which the ratio remained constant = 5, and number of responses by which the ratio increased per increment = 3, i.e. on trials 1-5, 1 response

was required, on trials 6-10, 4 responses, on trials 11-15, 7 responses, and so on. If there was no single response within any 600-s period the break point was reached and the session terminated. Measures were total number of nosepokes, number of reinforcements attained, final ratio attained, and total number of responses at the feeder. In Study B, at 100% baseline BW, mice were given two PRS tests on consecutive days, and the scores on the second day were used to counter-balance allocation of mice to CSD and CON groups in terms of reward motivation (Fig. 1B). Mice were tested at 100% baseline BW for CSD effects in the PRS test at CSD days 8 and 15, with testing conducted, before the daily CSD session, at 09:00-12:00 h. Within CSD and CON mice, PRS behaviour at CSD day 8 was used to counter-balance allocation of mice to AGO and VEH drug groups.

Simple reversal learning (SRL) test. In Studies A and B, mice were trained on reversal learning, using two nose-poke apertures positioned left and right of the central feeder. The final parameters for reversal training, and also the parameters used in the SRL test itself, were: On FR1, a response at the correct aperture initiated tone and pellet delivery followed by 2.5 s ITI, and a response at the incorrect aperture initiated 5-s time out. Consistent reward-stay behaviour in the form of eight consecutive correct responses/reinforcements was required for reversal. At reversal the previously incorrect aperture was now correct and *vice versa*, so that nonreward-shift behaviour was required. The total number of pellets available was 48, giving a maximum of six reversals. Criteria for completion of reversal-test training were all 48 reinforcements obtained and consumed, a minimum of 18 ($p \geq 0.75$) reward-stay responses per aperture, and a minimum of 3 reversals completed. Mice required 35-40 sessions, 5 days per week, from onset of operant training to reach these criteria for reversal learning. The final settings described above were also the settings for the actual SRL test. The measures of interest were as described below for the complex reversal test, except there was no measure, negative feedback sensitivity.

Complex reversal learning (CRL) test. The CRL test was used in Studies A and B, and was conducted as reported elsewhere (Ineichen *et al.*, 2012) where the same test was described as probabilistic reversal learning. (Given that in "real" probabilistic reversal learning tests both a proportion of correct responses are not rewarded and a proportion of incorrect responses are rewarded (e.g. Bari *et al.* (2010), whereas in the present test only the former contingency was used, we use the term complex reversal learning test.) Briefly, the same parameters as in the SRL test were used with the addition of an overall probability of 0.15 that responses to the correct aperture were not rewarded; these were called non-rewarded correct responses (NR-CRs). The first correct response per session was always rewarded and the maximum number of consecutive NR-CRs was 2. A maximum of 60 pellets could be obtained and the maximum amount of reversals was 7 therefore. Measures of

interest were: *Reward-stay probability*, calculated as *p* (trials with response to the aperture that was correct on previous trial/total trials immediately following a correct trial). *Number of reversals completed*. *Perseverations/reversal*, immediately following reversal, the number of consecutive trials required to switch to the new correct aperture /total reversal completed. *Trials/reversal*, mean number of trials required to complete a reversal. *Negative feedback sensitivity*, calculated as *p* (trials with response to the opposite aperture following a NR-CR/total trials immediately following a NR-CR). *Average latency to collect reward*, mean time (ms) required to collect the pellet from the feeder. *Session duration*, the time (s) needed to finish the session by obtaining 60 pellets or 1800 s maximum.

In Study A, all mice were given five CRL tests, each preceded by a different compound (see section Drugs below). Mice were given the SRL test on days between CRL tests, to ensure that they were exhibiting normal baseline performance on the day prior to compound-CRL testing (Fig. 1).

In Study B, following CSD (day 15) mice were returned to food restriction on day 16 to reduce BW to 95% baseline. They were given a SRL test on day 19, and a CRL test on day 22; these were days 10 and 13 of daily AGO/VEH administration, respectively (Fig. 1, section Drugs below).

IntelliCage appetitive and consummatory behaviour. In Study C (Fig. 1C), IntelliCage (TSE) was used, a system for automated continuous monitoring of appetitive and consummatory behaviour of mice in their home cage, as described elsewhere (Cathomas *et al.*, 2015a; Cathomas *et al.*, 2015b). Briefly, each IntelliCage was divided at the centre by a transparent, perforated divider to give two independent compartments, and placed in an attenuation chamber with a 12:12 h reversed light-dark cycle (light off 07:00-19:00 h). Mice were fitted with a subcutaneous transponder to record: visits to the operant devices located in each corner; operant nosepokes into a light sensor aperture at the door in each corner that opened on FR1 to allow access to a drinking bottle; and the number of licks, measured by electrical contact of the tongue with the drinking tip. Littermate pairs were habituated to the cage for 3 days with operant doors open, and then the doors were closed so that mice had to nosepoke to open a door and access the water bottle for 20 s, for a training period of 7 days. This was followed by 5 days of training with one water bottle and one saccharin solution (0.1%, sodium salt hydrate, Sigma) bottle, the corner locations of which were alternated each day at 17:00 h. For 10-day baseline data collection, the littermate pair was separated with one mouse placed in each compartment, and they were exchanged between compartments each day at 17:00 h. In the first experiment (Fig. 1C1), this continued for a further 15 days in the case of CON mice. In CSD mice, one littermate was removed and replaced by a CD-1 mouse. The CSD procedure was conducted at 16:00-17:00 h for 15 days. Thus CSD immediately preceded the time of exchanging compartments and bottle locations during baseline data collection: the CSD mouse was placed in the compartment

occupied by the CD-1 mouse for either a cumulative total of 60-s physical attack or 10 min maximum, and then the CSD mouse remained in this compartment and the CD-1 mouse was transferred to the opposite compartment. CD-1 mice were provided with a normal water bottle; they were rotated between IntelliCages every fifth day and lower incisors were trimmed every third day. In the second experiment (Fig. 1C2), after the 10-day baseline data collection, the CSD procedure was conducted on days 1-15. On days 16-20, the CSD and CD-1 mice were exchanged between compartments at 16:00 h without further attacks. As in Study B, the rationale for this was that after 15 proximate exposures to CD-1 mice, distal exposure to such a mouse would continue to represent a stressor until the end of the experiment. AGO/VEH were administered at days 7-20, at 06:00-06:30 (see section 2.6). For statistical analysis, four time blocks of interest were identified: two during the light phase, 20:01-00:00, 00:01-04:00, and two during the dark phase, 08:01-12:00, 12:01-16:00. Therefore, the data collected during the 3-4 hours directly after CSD/CON and compartment transfer were not included in the statistical analysis, as was also the case for the data collected during the 2 hours after AGO/VEH administration. This was done to avoid inclusion of any acute effects of CSD, compartment transfer or compound administration in the data set, with the focus being on analysing for sustained effects of CSD and AGO, and during both the dark phase and light phase.

2.6. Drugs

To establish dose and time effects on AGO levels, naive mice (N=6-7 per dose x time point) were orally (*p.o.*) administered 10 or 25 mg/kg AGO using intragastric gavage, and trunk blood and brain were collected after 1 h or 3 h and stored at -80°C prior to analysis. AGO measurement in plasma and brain-tissue homogenate was performed according to an established method involving liquid-liquid extraction of a sample volume of 20 µl, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) detection. S 40706-1 (D5-20098) was used as an internal standard. The limit of quantification (LLOQ) was 0.100 ng/mL (range 0.100 to 30 ng/mL). All LC-MS/MS measurements were performed by a central analytical laboratory.

All compounds were prepared fresh in a vehicle of 1% hydroxyethylcellulose (HEC) in water. In Study A, acute AGO was studied at 10 and 25 mg/kg *p.o.*, acute melatonin (MT) at 10 mg/kg *p.o.*, and the 5-HT_{2C} antagonist S 32006 acutely at 2.5 mg/kg *p.o.* The melatonin dose of 10 mg/kg was based on previous demonstrations of efficacy of this dose in preventing stress-induced behavioural effects in mice (e.g. Gumuslu *et al.*, 2014). The S 32006 dose of 2.5 mg/kg was based on previous demonstration of antidepressant- and anxiolytic-like effects of this dose in rat and mouse models and tests (Dekeyne *et al.*, 2008), and on pilot study evidence that 5 and 10 mg/kg increased session duration in reversal tests suggesting inhibition of responding. A latin square design was used to test compound effects in a counterbalanced order across mice. A period of 3-4 days was allowed

between each day of compound-CRL testing (Fig. 1A). In Study B, 13-day repeated AGO was studied at 25 mg/kg *p.o.*. In Study C, 14-day repeated AGO was studied at 25 mg/kg *p.o.*. Compounds were administered at the end of the light phase (06:00-06:30 h) in all studies; 1-2 hours prior to operant testing in Studies A and B, and 2 hours prior to onset of collection of data used in the statistical analysis in Study C.

2.7. Statistical analysis

Statistical analysis was conducted using SPSS (version 21, SPSS Inc., Chicago IL, USA). In Study A, drug effects on dependent measures in the CRL test were analysed using repeated measures analysis of variance (ANOVA). In Study B, CSD effects on food required to maintain baseline BW at specific time points were studied using *t*-tests. 2 Stress (CSD, CON) x 2 Drug (AGO, VEH) full-factorial ANOVA was used to study effects on behavioural measures in the PRS, SRL and CRL tests. In Study C, in the first experiment 2 Stress (CSD, CON) x 4 Day-block (Baseline, CSD/CON 1-5, 6-10, 11-15) x 4 Time-block (20-00 h, 00-04 h, 08-12 h, 12-16 h) mixed-factorial ANOVA was used to study effects on IntelliCage measures; and in the second experiment 2 Drug (AGO, VEH) x 5 Day-block (Baseline, CSD 1-5, CSD-Drug 6-10, CSD-Drug 11-15, Drug 16-20) x 4 Time-block (20-00 h, 00-04 h, 08-12 h, 12-16 h) ANOVA was used to study effects on IntelliCage measures. Significant main or interaction effects were analysed using *post hoc* testing with Bonferroni correction for multiple comparisons. Significance was set at $p \leq 0.05$ and a non-significant trend at $0.05<p<0.10$. Effect size values were calculated as Cohen's d for *t*-test and partial eta squared (η^2) for ANOVA. Data are given as means and where an estimate of variance is given this is the standard deviation (SD).

3. Results

3.1. Agomelatine pharmacokinetics

In naive mice, agomelatine blood and brain levels following *p.o.* administration at 10 or 25 mg/kg and sample collection after 1 or 3 h are given in Figure 2. In plasma there was a Dose x Time interaction effect on AGO levels ($F(1, 20)=9.52$, $p<0.006$, partial $\eta^2=0.32$, Fig. 2A) and the same was the case for whole brain levels ($F(1, 22)=4.34$, $p<0.05$, partial $\eta^2=0.17$, Fig. 2B). In each compartment, AGO levels were increased at 1 h after administration of 25 mg/kg *p.o.* relative to all other Dose x Time points, at which AGO levels were detectable and low.

FIGURE 2 ABOUT HERE

3.2. Study A: Effects of acute agomelatine in the complex reversal learning test

In naive mice in the CRL test, each mouse was tested following acute *p.o.* AGO at 10 mg/kg (AGO 10) or 25 mg/kg (AGO 25), melatonin (MT) at 10 mg/kg, and S 32006 (5-HT_{2C} ANT) at 2.5 mg/kg, using a latin square design. As given in Figure 3, there was no Drug effect on reward-stay behaviour (p=0.30, Fig. 3A), and also no Drug effect on negative feedback sensitivity (p=0.40, Fig. 3B). For number of reversals completed, there was a main effect of Drug (F(4, 114)=3.06, p<0.05, partial η^2=0.11, Fig. 3C); post hoc testing demonstrated that, relative to VEH, mice completed more reversals after AGO 25 (p<0.05) and 5-HT_{2C} ANT (p<0.03).

Based on both the pharmacokinetic and CRL test data, 25 mg/kg AGO was the dose selected for repeated oral administration in Studies B and C.

FIGURE 3 ABOUT HERE

3.3. Study B: Effects of CSD and repeated agomelatine on reward motivation and response accuracy in discrete operant tests

CSD effects on feeding homeostasis and appetite hormones

Prior to Study B, in a separate cohort of naive mice we investigated CSD effects on food intake under *ad libitum* conditions and plasma levels of appetite-regulating hormones. CSD mice exhibited a marked increase in food intake: mean daily weight of pellet eaten was calculated for the 5-day blocks, pre-CSD/CON -4 to 0, CSD/CON 1-5, 6-10, 11-15. There was a Group x Day-block interaction (F(3,60)=13.95, p<0.0005, partial η^2=0.54 Fig. 4A); pre-CSD/CON food intake was similar in CON and CSD mice, and CSD mice consumed more pellet than did CON mice at days 1-5, 6-10 and 11-15. For body weight, there was no effect of Group (p≥0.61, Fig. 4B), and a main effect of Day-block (F(3,60)=21.83, p<0.0005, partial η^2=0.52, Fig. 4B) due to a general increase in BW over time. The increase in food intake in CSD mice co-occurred with a decrease in plasma levels of the appetite-suppressant hormone leptin in samples collected on day 16 (CON: 5.24±2.26 ng/ml, CSD: 2.12±0.93 ng/ml, $t_{(21)}$=4.77, p<0.0002, d=1.95). There was no overall effect of CSD on plasma levels of the appetite-enhancing hormone ghrelin in the same mice (CON: 75.90±28.42 pg/ml, CSD: 107.80±70.24 pg/ml, p=0.19). These findings demonstrated the important need to control the BW and pellet allocation for each mouse individually and on a daily basis, in order to maintain all mice at the same BW relative to baseline, and therefore in as homeostatic state as possible, for operant testing.

FIGURE 4 ABOUT HERE

CSD and agomelatine effects in the progressive ratio schedule test

In Study B, conducted with naive mice, with respect to baseline BW and pellet intake, mice (N=22) that were later allocated to CON weighed 28.2±1.62 g (=100% baseline BW) and consumed 3.8±0.4 g pellet/day, and mice (N=22) that were later allocated to CSD weighed 28.7±1.6 g and consumed

3.8±0.5 g pellet/day. During the 15-day CSD/CON period, CON mice were given 3.1±0.5 g food pellet/day to maintain 100% baseline BW (actual values: 101.4±2.2%), whereas CSD mice needed to be given 4.7±0.7 g food pellet/day to maintain 100% baseline BW (actual values: 101.5±1.5%). Therefore, as observed in the ad libitum food intake experiment (Fig. 4), CSD mice required more food to maintain body weight.

At CSD/CON day 8, which was prior to commencement of AGO administration (day 10, Fig. 1), mice were given a PRS test. At this stage there was no effect of CSD on the main measures of total number of operant responses (CON: 273±236, CSD: 281±177, p=0.91), reinforcements attained (CON: 27.4±11.8, CSD: 28.5±10.0, p=0.72), and final ratio attained (CON: 16.4±7.1, CSD: 17.4±5.8, p=0.63). However, the number of responses at the feeder was decreased in CSD mice (CON: 164±88, CSD: 110±68, $t_{(42)}$=2.33 p<0.03, d=0.71), suggesting decreased reward anticipation. Within CON and CSD groups, the total number of PRS responses was used to counter-balance allocation of mice to AGO and VEH groups.

At CSD/CON day 15, which was day 6 of AGO administration, a further PRS test was conducted. At this stage, there was a consistent effect of CSD on PRS test behaviour. Thus, for total number of operant responses there was a main effect of Stress group (F(1,40)=11.44, p<0.002, partial η^2=0.22, Fig 5A) with CSD mice making less responses. CSD mice also attained fewer reinforcements (p<0.001, partial η^2=0.24, Fig 5B) and a lower final ratio (p<0.001, partial η^2=0.24, Fig. 5C) relative to CON mice. The number of responses at the feeder was also lower in CSD mice (CON: 152±68, CSD: 69±43, p<0.0005, partial η^2=0.24), suggesting decreased reward anticipation. With the exception of three CSD-VEH mice which reached break point, all mice continued responding to the end of the 40-min PRS session. There was no Stress x Drug interaction or main effect of Drug for any PRS measure. However, as is evident in each data set in Figure 5, in CSD mice specifically there was an increase in the mean scores in mice receiving AGO; in t-tests, the final ratio attained in CSD-AGO versus CSD-VEH mice tended to increase without reaching statistical significance (p=0.07, d=0.81, Fig. 5C). There was no effect of AGO on the amount of food that needed to be given to maintain mice at 100% baseline BW, in either CSD or CON mice.

FIGURE 5 ABOUT HERE

CSD and agomelatine effects in the reversal learning tests

The same mice were then reduced to 95% baseline BW (CON: 96.2±2.2%, CSD: 97±3.3%, p=0.37) and studied in terms of CSD and AGO effects on reversal learning. At day 19, 4 days after the end of CSD and day 10 of AGO administration, mice were tested in the simple reversal learning test (Figure 6). There were main effects of Stress group, consistent with impaired test performance in CSD mice, for: number of reinforcements obtained (CON: 48±0, CSD: 40±13, p<0.007, partial η^2=0.17), probability

of reward-stay responding (p<0.0005, partial η^2=0.34, Fig. 6A), reversals completed (p<0.0005, partial η^2=0.27, Fig. 6B), trials per reversal (p<0.003, partial η^2=0.19, Fig. 6D), average latency to collect reward (p<0.006, partial η^2=0.17, Fig. 6E) and session duration (p<0.02, partial η^2=0.15, Fig. 6F). There was no effect of CSD on perseverations per reversal (p=0.27, Fig. 6C). There was no Stress X Drug interaction or main effect of Drug for any SRL measure.

FIGURE 6 ABOUT HERE

At day 22, mice were tested in the complex reversal learning test (Figure 7). There were main effects of Stress group, consistent with impaired test performance in CSD mice, for: number of reinforcements obtained (CON: 60±1, CSD: 52±11, p<0.002, partial η^2=0.21), probability of reward-stay responding (p<0.004, partial η^2=0.19, Fig. 7A), reversals completed (p<0.001, partial η^2=0.23, Fig. 7B), trials per reversal (p<0.03, partial η^2=0.12, Fig. 7D), average latency to collect reward (p<0.04, partial η^2=0.10) and session duration (p<0.0005, partial η^2=0.34, Fig. 7F). Negative feedback sensitivity was actually reduced in CSD mice relative to CON mice (p<0.02, partial η^2=0.14, Fig. 7E). In addition to the CSD effect on reversals completed, there was also a borderline non-significant effect of Drug (p<0.08, partial η^2=0.07, Fig. 7B); in Stress group-specific t-tests, CSD-AGO mice completed more reversals than did CSD-VEH mice (p<0.03, d=0.93, Fig. 7B). For perseverations per reversal, there was a Stress x Drug interaction (p<0.03, partial η^2=0.12, Fig. 7C), and *post hoc* tests identified that CSD-AGO mice perseverated more than each of the other three groups.

FIGURE 7 ABOUT HERE

3.4. Study C: Effects of CSD and repeated agomelatine on reward motivation and circadian rhythm in the home cage

In the first IntelliCage study, conducted with naive mice (Fig. 1C1), saccharin- and water-directed visits, nosepokes and licks, were compared in CSD and CON mice (Figure 8 and Table S1). Baseline (days -4 to 0) behaviour was similar in mice allocated at random to CON and CSD groups (Fig. 8A-D, Table S1); as expected (e.g. (Cathomas *et al.*, 2015a)), under baseline conditions and primarily during the dark phase, mice had a moderate preference for the saccharin corner versus water corner in terms of visits and nosepokes, and a marked preference for saccharin over water in terms of licks. In mixed-factorial ANOVA, in cases of significant interaction of Stress x Day x Time and/or Stress x Day and/or Stress x Time, *a posteriori* Stress x Time analysis was conducted for each day-block separately. For saccharin visits (Stress x Day x Time p<0.03, partial η^2=0.12), CSD mice made less saccharin visits than CON mice during the dark phase at day-blocks CSD 1-5, 6-10 and 11-15 (p<0.01-0.05, Table S1). A similar CSD effect pertained for water visits (Stress x Day x Time p<0.0005, partial η^2=0.21), with CSD mice making less visits than CON mice during the dark phase at day blocks CSD 1-5, 6-10 and

11.15 (p<0.01-0.05, Table S1), and also CSD mice making more water visits than CON mice during the light phase at day-blocks CSD 6-10 and 11-15 (p<0.01-0.05, Table S1). Together, these data suggest a general decrease in goal-directed dark-phase activity in CSD mice. For saccharin nosepokes (Stress x Day x Time p<0.02, partial η^2=0.13), CSD mice made less saccharin nosepokes than CON mice during the dark phase at day-blocks CSD 1-5, 6-10 and 11-15 (p<0.05, Fig. 8A, Table S1). A similar CSD effect pertained for water nosepokes (Stress x Time p<0.03, partial η^2=0.31), with CSD mice making less nosepokes than CON mice during the dark phase at day-blocks 1-5, 6-10 and 11-15 (p<0.05-0.01, Fig. 8B, Table S1), again consistent with a general decrease in appetitive behaviour. For saccharin licking (Stress x Day x Time p<0.05, partial η^2=0.11, Fig. 8C, Table S1), CSD mice made less saccharin licks than CON mice during the dark phase at day-block CSD 11-15, consistent with decreased interest in saccharin by this stage (p<0.05, Fig. 8C, Table S1). For water licks (Stress x Day x Time p<0.0005, partial η^2=0.19, Fig. 8D, Table S1), CSD mice made more licks than CON mice during the light phase at day-blocks CSD 1-5 and 6-10 (p<0.001-0.01, Fig. 8D, Table S1), possibly reflecting increased activity including feeding during this period.

In the second IntelliCage study, conducted with naive mice (Fig. 1C2), in which CSD-AGO and CSD-VEH mice were compared (Figure 9A-D and Table S2), the saccharin- and water-directed behavioural profiles of these mice were similar to those of the CSD mice in the first experiment. This was indicated by the main effect of Day-block in the Drug x Day-block x Time-block ANOVA, attributable to a decrease in behaviour relative to baseline (days -4 to 0) in at least one CSD/post-CSD day-block, for dark-phase saccharin visits, dark-phase saccharin nosepokes (see Fig. 9A) and dark-phase saccharin licks (see Fig. 9C). Furthermore, light-phase water licks were increased in CSD/post-CSD day-blocks relative to baseline (see Fig. 9D). There were no significant effects involving Drug for any behavioural measure in these CSD mice.

FIGURE 8 ABOUT HERE

FIGURE 9 ABOUT HERE

4. Discussion

In the present study we could demonstrate that exposure of mice to chronic psychosocial stress leads in discrete operant tests to: decreased motivation to exert physical effort for gustatory reinforcement, and to decreases in both response accuracy and negative feedback sensitivity when cognitive effort is required to obtain gustatory reinforcement. Furthermore, in the home cage, appetitive and to a lesser extent consummatory responding to gustatory reward were decreased. At a pharmacological dose, the antidepressant agomelatine was effective in reducing the stress-induced deficit in complex reversals completed, and this was associated with an increase in perseveration which was adaptive under the test conditions. ~~Given that this is the first study to investigate CSD~~

~~effects on operant reward behaviour, t~~These findings add considerably to the evidence that mouse models relevant to stress-induced disruption of the motivation and cognitive flexibility to interact with rewarding stimuli, core and common psychopathology domains in depression, can be developed. Furthermore, the partial reversal of these deficits by the antidepressant AGO provides some evidence for the predictive validity of the model.

4.1. Positive agomelatine and 5-HT_{2C}-antagonist effects on complex reversal learning

In the complex reversal learning (CRL) test, an acute dose of AGO at 25 mg/kg or of a 5-HT_{2C} antagonist, administered orally 1-2 hours prior to testing, led to an increase in the number of reversals completed by otherwise non-manipulated mice; no effect was observed with AGO at 10 mg/kg or melatonin in the same experiment. Although neither measure responded significantly, AGO (25 mg/kg) and S32006 did result in a mean increase in reward-stay probability and a mean decrease in negative feedback sensitivity (NFS), suggesting that these two effects contribute additively to the significant drug effects on reversals completed. In a previous study, a low, acute dose of the antidepressant escitalopram increased reward-stay, decreased NFS and increased reversals completed in mice in this CRL test (Ineichen *et al.*, 2012). In healthy humans, 3-day AGO administration led to increased reward-stay probability in probabilistic reversal learning tests compared with vehicle and escitalopram (Salvador *et al.*, 2014). In a simple reversal learning test in rat, another 5-HT_{2C} antagonist, SB 242084, also improved reversal learning (Boulougouris *et al.*, 2008). 5-HT_{2C} receptors are expressed in a number of forebrain regions important in the regulation of operant behaviour, including frontal cortex, amygdala, hippocampus, and dorsal and ventral striatum. 5-HT_{2C} antagonism disinhibits dopamine (DA) release into frontal cortex specifically (Di Giovanni *et al.*, 2006; Millan *et al.*, 2003), as demonstrated after AGO administration in rats (Millan *et al.*, 2003). 5-HT_{2C} receptors are also expressed in monoamine cell-body regions, including ventral tegmental area (VTA) for DA and locus coeruleus for noradrenaline (NA) (Pompeiano *et al.*, 1994; Racagni *et al.*, 2011). In VTA, 5-HT_{2C} receptors are expressed by GABA interneurons that project onto DA neurons (Eberle-Wang *et al.*, 1997). AGO, acute and chronic, increases the number of spontaneously active VTA DA neurons and the bursting activity of VTA dopaminergic neurons (Chenu *et al.*, 2013). Nonetheless, 5-HT_{2C} antagonism does not disinhibit DA release in nucleus accumbens or dorsal striatum (Millan *et al.*, 2003).

4.2. CSD-induced decreased motivation and cognitive deficits for operant-reward

Chronic social defeat resulted in increased food intake, as described previously (Kumar *et al.*, 2013). This occurred in the absence of an effect on body weight, consistent with CSD inducing an increase in energy expenditure. In blood samples collected on day 16 from these same mice, there was a

decrease in plasma leptin levels, as reported previously for CSD (Kumar *et al.*, 2013). Leptin is an adipokine released from fat into the circulation. It is transported across the blood-brain-barrier where it acts to suppress feeding. It acts at homeostatic areas (e.g. brainstem, hypothalamus) as well as areas involved in reward-motivation such as VTA. The reduction in plasma leptin observed in CSD mice relative to CON mice under free-feeding conditions would be expected to contribute to their increased feeding by disinhibition. There was no consistent effect of CSD on the active form of ghrelin, which acts to promote feeding and has been reported to be increased by CSD and to underlie the increased feeding by CSD mice that, in contrast to the present study, exhibited increased body weight (Patterson *et al.*, 2013). These findings confirmed the importance of providing CSD and CON mice with the group- and individual-specific feeding regimens that were required for maintaining the required level of baseline body weight, in order to ensure that all mice were in as similar state as possible for operant testing.

Chronic social defeat was without effect on reward motivation in the progressive ratio schedule (PRS) test at CSD day 8, but had resulted in decreased reward motivation under these effortful conditions relative to CON mice, as indicated by the inter-related reductions in number of responses and rewards and final ratio attained, by the final day, 15, of CSD. In human depression, patients do not differ from healthy controls in their reports of the intensity and pleasantness of sucrose, indicating that hedonic reactivity to reward consumption is intact (Dichter *et al.*, 2010). However, using monetary reward, depressed patients exhibit reduced motivation to expend effort to obtain reward (Treadway *et al.*, 2012). In the same mice, testing in the simple reversal learning (SRL) test at day 19 and the CRL test at day 22 identified further disruption of reward processing in mice that had experienced CSD. Thus, in the SRL test CSD mice exhibited reduced reward-stay accuracy, completed less reversals, and required more trials per reversal, relative to CON mice. That these effects co-occurred in CSD mice with reduced reward motivation – despite testing being conducted at 95% baseline body weight - was indicated by their increased latency to collect the sucrose-pellet after a correct response. The CSD-induced deficits in the SRL test also pertained in the CRL test at day 22. That performance in terms of reward-stay accuracy and reversals completed was reduced in both CON and CSD mice in the CRL relative to the SRL test, confirms the increased cognitive demands of the former test. In addition to reduced motivation, reduced ability to accurately monitor the correct operant stimulus (i.e. nose poke aperture) under cognitively effortful conditions and reduced reward expectancy, are two further potential deficits that could account for the CSD effects observed in these tests. In the CRL test specifically, negative feedback sensitivity was assessed, and interestingly CSD actually caused a decrease in NFS. Non-manipulated mice, relative to healthy humans, exhibit high NFS in this type of test: we have interpreted this as evidence that mice are indeed able to monitor reward expectancy in the CRL test, experience the withholding of an expected

reinforcement as aversive and, in contrast to healthy humans, cannot routinely inhibit switching to the opposite operant stimulus on the next trial (Ineichen *et al.*, 2012). Against this background, the reduced NFS in CSD mice could indicate reduced cognitive ability to monitor reward expectancy, reduced negative emotionality when an expected reinforcement is withheld, or reduced reward expectancy. In the human PRL test, depressed patients exhibit intact reward-stay accuracy and reversals completed in combination with increased NFS, relative to healthy controls (Taylor Tavares *et al.*, 2008). Therefore, the constellation of CSD effects in the mouse CRL test is clearly not a recapitulation of the effects of depression in the human PRL test. Despite this, the mouse CSD-CRL test model comprises stress-induced deficits in reward-directed behaviour under cognitively demanding conditions, and this is certainly of high relevance to the major depression psychopathology of reduced motivation and interest in daily activities.

Evidence that CSD also causes continuous reduction in motivation to engage in appetitive behaviour in a home cage setting, complementary to that obtained in the discrete testing conditions of the PRS test, was provided by the IntelliCage experiment. CSD mice developed reduced goal-directed behaviour (visits, nosepokes) for saccharin and water during the active period of their circadian cycle from the first days of CSD. Towards the end of CSD they had also developed reduced consumption (licks) of saccharin during the active period. During the inactive period, the water consumption of CSD mice increased, possibly indicating that they were more active than CON mice during this period and eating more food, which stimulates drinking.

Using a different CSD protocol (10 days) to that of the present study, in some studies CSD mice have been divided into susceptible and resilient sub-groups based on a social interaction test in which social avoidance of a mouse of the strain (CD-1) used for CSD is measured (e.g. Krishnan *et al.*, 2007). Susceptible mice are defined as those that exhibit increased avoidance of the CD-1 mouse compared to controls, and resilient mice as those that exhibit avoidance overlapping with that shown by control mice. By categorising mice in this way it has been possible to demonstrate behavioural effects of CSD in some other tests specific to the susceptible group e.g. decreased sucrose preference (Krishnan *et al.*, 2007). Using our 15-day CSD protocol and treating CSD mice as one group we have obtained significant CSD effects in terms of increased fear conditioning, learned helplessness and fatigue (Azzinnari *et al.*, 2014; Fuertig *et al.*, 2016), and now in the present study in terms of reward-directed operant behaviour in motivation and cognitive tests. This "inclusive" experimental design has been used extensively with other stressors, such as chronic unpredictable mild stress (e.g. Tye *et al.*, 2013; Willner, 1997).

4.3. Partial reversal of CSD decreased motivation and cognitive effects with repeated agomelatine

Agomelatine, using the dose of 25 mg/kg at which acute administration led to increased reversals completed in the CRL test in non-manipulated mice, exerted some normalising effects on operant behaviour when administered repeatedly in CSD mice. Firstly, there was a tendency to increase motivation for reward in the PRS test conducted after 6 days of repeated AGO, in CSD mice. A similar reduction in motivation in the PRS test to that observed in CSD mice is induced by 6-hydroxydopamine depletion of DA in the nucleus accumbens (Bergamini *et al.*, in press ~~Submitted manuscript~~). Extrapolating to the current findings, this suggests that AGO effects on the VTA-DA pathway mediated via it 5-HT_{2C} antagonist properties (Chenu *et al.*, 2013) could have contributed to the increased motivation in CSD-AGO mice. Whilst there were no effects of 10 days of AGO in the SRL test, 13 days of AGO resulted in an increase in reversals completed in the more demanding CRL test in CSD mice specifically. Furthermore, this co-occurred with an increase in the average number of perseverations per reversal in CSD-AGO mice compared with each of the other groups. In the CRL test, where there are non-rewarded correct responses (NR-CRs) that could be interpreted - incorrectly - as initiation of reversal, it is actually adaptive to be perseverative because this allows for differentiation between non-rewarded correct response trials and onset of reversal trials. Two perseverative responses per NR-CR/reversal are sufficient in this respect: CSD-AGO mice made a mean of 2-3 perseverations per reversal and each of the other groups made a mean of 1-2 perseverations per reversal. This co-occurred with CSD-AGO mice also exhibiting the lowest mean NFS score of all groups. Therefore, repeated AGO produced increased perseveration specifically in CSD mice and specifically in the CRL test where, in contrast to the SRL test, some perseveration is adaptive. This might represent a first indicator of normalisation of reward expectancy and motivation in CSD mice, due to repeated administration of AGO. With regards to underlying mechanisms, the prefrontal cortex and nucleus accumbens are major regions in the regulation of perseveration (Clarke *et al.*, 2004; Cools *et al.*, 2007). In rat, the 5-HT_{2C} antagonist SB 242084 decreased perseveration in a reversal learning test (Boulougouris *et al.*, 2008), suggesting that the current increase in perseveration in AGO-treated CSD mice might not be directly related to 5-HT_{2C} antagonism. Also in rat, infusion of the D_2 receptor antagonist eticlopride into the PFC increased perseveration (Floresco *et al.*, 2006). In Parkinson's disease patients, L-DOPA increases perseveration in a probabilistic reversal learning test, associated with disrupted activity related to excessive DA signalling in the nucleus accumbens (Cools *et al.*, 2001; Cools *et al.*, 2007). 5-HT_{2C} receptor antagonists and AGO increase DA release into frontal cortex (Di Giovanni *et al.*, 2006; Millan *et al.*, 2003) and AGO increases bursting activity of VTA dopaminergic neurons (Chenu *et al.*, 2013). Therefore, the AGO-related increase in perseveration specific to CSD mice could reflect increased sensitivity to up-regulated DA signalling following a prolonged period of low DA tone in the mesocorticolimbic pathway. It has been demonstrated that chronic stress leads to decreased activity of VTA neurons in

mice (Tye *et al.*, 2013). Despite the evidence that 5-HT_{2C} antagonism might stimulate appetite for food (Voigt and Fink, 2015), the absence of AGO effects on operant behaviour in CON mice indicates the absence of any such confounding effect in the present study.

There were no effects of AGO on the CSD-induced deficits in appetitive behaviour and shifts in circadian activity in IntelliCage. This indicates that the observed effects of AGO were specific to reward processing occurring under demanding test conditions, namely high physical effort in the PRS test or high cognitive effort in the CRL test. Reinstatement of typical levels of maintenance and reward-directed behaviour under low-effort/cognition conditions might require a longer duration of AGO exposure.

4.4. Conclusion

~~To our knowledge, this is the first study to investigate the effects of mouse CSD on reward processing in operant tests. P~~In this study, psychosocial defeat stress has been demonstrated to lead to behavioural changes consistent with decreases in reward motivation, stimulus-reward monitoring and reward expectancy. Furthermore, CSD increased activity during the inactive phase of the circadian cycle. Given that these behavioural processes are directly relevant to the RDoC domain of positive valence systems, and similar deficits have been reported in depressed patients, the model established here demonstrates aetiological and face validity. Partial reversal of these deficits by the antidepressant agomelatine provides some evidence for the model's predictive validity. Future studies with the model will investigate the neurobiological underpinnings of the stress-induced deficits in reward processing.

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Contributors

GB, FC and MP conducted the study, analysed the data and wrote the manuscript. SA and HS conducted the study. ES wrote the manuscript. CG designed the study and wrote the manuscript. CRP designed the study and wrote the manuscript.

Conflict of interest

Cecilia Gabriel is a Servier employee. All other authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Experimental designs. (A) Study A: Latin square design to study effects of acute agomelatine, melatonin and S 32006, a 5-HT_{2C} receptor, antagonist on behaviour in a complex reversal learning test in otherwise non-manipulated mice. (B) Study B: Effects of chronic social defeat versus control handling and AGO versus vehicle on behaviour in a progressive ratio schedule test and simple and complex reversal learning tests. (C) Study C: Experiment 1, effects of CSD versus CON handling on appetitive and consummatory behaviour towards water and saccharin solution during the dark- and light-phases of the circadian cycle. Experiment 2, effects of AGO versus vehicle on these same measures in CSD mice. Baseline BW/b-BW: baseline body weight; FI: Food intake; SRL: simple reversal learning test; CRL: complex reversal learning test; D: acute drug; CSD: chronic social defeat; CON: control handling; PRS: progressive ratio schedule test; AGO: agomelatine; VEH: vehicle.

Figure 2. Agomelatine concentrations in (A) trunk blood plasma and (B) whole brain tissue in the same naive mice following oral administration of agomelatine at 10 or 25 mg/kg at the end of the light phase and sample collection after 1 or 3 hours. N=6-7 mice per dose and time point. In (A) and (B), there was a Drug dose x Time interaction in ANOVA, and dose/time points with different letters (a vs b) were different (p<0.005) according to pairwise Bonferroni testing. Note the different Y-axis scales used in (A) and (B).

Figure 3. Compound- and dose-dependent effects of vehicle, agomelatine (AGO, 10 and 25 mg/kg), melatonin (MT, 10 mg/kg) and S 32006, a 5-HT_{2C} antagonist (5-HT2C ANT, 2.5 mg/kg) on behaviour in the complex reversal learning (CRL) test. The test was conducted 1-2 hours after *p.o.* compound had been administered at the end of the light phase. A latin square design for the order of compound/dose testing was used. Twenty four mice were studied, and each mouse obtained the maximum 60 reinforcements in each test session. A) Probability of reward-stay responding. B) Negative feedback sensitivity (NFS) with respect to switching stimulus after a non-rewarded correct response. C) Number of reversals. Values are mean $\pm$ SD. * p<0.05 for post hoc Bonferroni testing conducted after a main effect of Drug.

Figure 4. Effects of chronic social defeat on mouse food intake and body weight. A) Mean food intake per 5-day block. B) Mean body weight per 5-day block. The weights of food given and remaining were measured each day at 16:00 h. Values are mean +/- SD. In A) *** p<0.001 for Bonferroni tests conducted for Stress group in specific Day-blocks following a Stress group x Day-block interaction in

ANOVA. In B) day-blocks with different letters (a vs b) $p<0.05$ or less for Bonferroni tests following main effect of Day-block.

Figure 5. Effects of chronic social defeat and repeated agomelatine (25 mg/kg *p.o.*) on mouse behaviour in the progressive ratio schedule test, at day 15 (final day) of CSD and after 6 days of AGO. The test was conducted 1-2 hours after AGO/VEH had been administered at the end of the light phase. Eleven mice per group were tested, counter-balanced with respect to baseline behaviour in this test. (A) Total number of nosepoke responses. (B) Number of reinforcements attained. (C) Final ratio attained. In A-C, *p* values are for the main effect of Stress group in a Stress group x Drug dose ANOVA. In C, in a Stress group-specific *t*-test, there was a borderline non-significant increase in the final ratio attained in CSD-AGO versus CSD-VEH mice.

Figure 6. Effects of chronic social defeat and repeated agomelatine (25 mg/kg *p.o.*) on mouse behaviour in the simple reversal learning test, at 4 days after completion of CSD and after 10 days of AGO. The test was conducted 1-2 hours after AGO/VEH had been administered at the end of the light phase. Eleven mice per group were tested. (A) Probability of reward-stay responding. (B) Number of reversals completed. (C) Perseverations per reversal. (D) Trials per reversal. (E) Latency to collect reward from feeder. (F) Session duration. *p* values are for the main effect of Stress group in a Stress group x Drug dose ANOVA.

Figure 7. Effects of chronic social defeat and repeated agomelatine (25 mg/kg *p.o.*) on mouse behaviour in the complex reversal learning test, at 7 days after completion of CSD and after 13 days of AGO. The test was conducted 1-2 hours after AGO/VEHhad been administered at the end of the light phase. Eleven mice per group were tested. (A) Probability of reward-stay responding. (B) Number of reversals completed. (C) Perseverations per reversal. (D) Trials per reversal. (E) Negative feedback sensitivity to non-reward of a correct response. (F) Session duration. In A, D-F, *p* values are for the main effect of Stress group in Stress group x Drug dose ANOVA. In B, there was a main effect of Stress group and borderline main effect of Drug group; Stress group-specific *t*-tests identified increased reversals due to AGO in CSD mice specifically ($p<0.03$). In C, there was a Stress group x Drug dose interaction, and *p* values are for Bonferroni pairwise tests ($p<0.01$).

Figure 8. Effects of chronic social defeat on mouse behaviour relative to saccharin and water, as measured continuously in IntelliCage. A) Saccharin nosepokes. B) Water nosepokes. C) Saccharin licks. D) Water licks. Seven CSD and 11 CON mice were studied; one mouse from each group had to be excluded because of technical problems. Each data point is the overall mean of 5-day-mean values

per mouse, obtained in 5 days prior to CSD/CON, CSD/CON days 1-5, 6-10 and 11-15, for each given 4-h period. Time periods were 20:00-00:00 and 00:00-04:00 during the light/inactive phase, 04:00-08:00 spanning the light-dark transition, and 08:00-12:00 and 12:00-16:00 during the dark/active phase. Daily CSD/CON procedures were carried out at 16:00-17:00 and the data obtained in the 3-4 h period thereafter were excluded from the analysis, as were the data obtained at 04:00-08:00. Following Stress group x Time period interaction, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ are for time-period specific Bonferroni tests of CSD versus CON mice. For additional data see Table S1.

Figure 9. Absence of effects of repeated agomelatine (25 mg/kg *p.o.*) in mice exposed to chronic social defeat on behaviour relative to saccharin and water, as measured continuously in IntelliCage. A) Saccharin nosepokes. B) Water nosepokes. C) Saccharin licks. D) Water licks. Eight CSD and 8 CON mice were studied. Each data point is the overall mean of 5-day-mean values per mouse, obtained in 5 days prior to CSD, on CSD days 1-5 without AGO/VEH, on CSD days 6-10 with AGO/VEH administration starting on day 7, on CSD days 11-15 with AGO/VEH, and on post-CSD days 16-20 with AGO/VEH, for each given 4-h period. Time periods were 20:00-00:00 and 00:00-04:00 during the light/inactive phase, 04:00-08:00 spanning the light-dark transition, and 08:00-12:00 and 12:00-16:00 during the dark/active phase. Daily CSD was carried out at 16:00-17:00 and the data obtained in the 3-4 h period thereafter were excluded from the analysis, as were the data obtained at 04:00-08:00. AGO/VEH were administered at 06:00-07:00. In Drug group x Time period interactions, there were no significant main or interaction effects that included Drug dose. For additional data see Table S2.